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Population Changes and Verotoxin Production of Enterohemorrhagic *Escherichia coli* Strains Inoculated in Milk and Ground Beef Held at Low Temperatures

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ABSTRACT

This study investigated the influence of low temperature and background flora on growth and verotoxin production by strains of enterohemorrhagic *Escherichia coli* in milk and ground beef. In the presence of no or low background flora, there was growth of the strains at 8°C. High background flora in ground beef inhibited growth at this temperature. In the foods held at low temperatures, only small amounts of verotoxin were detected; however, even at the optimum 37°C, there was still relatively little verotoxin formed compared to that in broth cultures. Even under nongrowth conditions (high background flora or 5°C holding temperature), the strains remained viable. These data suggest any food contaminated by these bacteria and held at the recommended temperature of 5°C will remain hazardous, and under certain conditions, holding at temperatures ≥8°C would increase the hazard.

Key words: *E. coli* O157:H7, verotoxin, background flora, low temperature, EHEC, milk, ground beef

Escherichia coli O157:H7 and other enterohemorrhagic *E. coli* have emerged as significant human pathogens, especially in young children. In addition to hemorrhagic colitis, these strains can also cause hemolytic uremic syndrome and thrombotic thrombocytopenic purpura; the Shiga-like toxins (verotoxins) produced by these strains can cause kidney damage and, in some instances, death *(11)*. While the exact incidence of these strains in the food supply is not known, their very low infective dose makes their presence at any level in a specific food a risk factor of major concern. To this point, these bacteria are most commonly associated with foods of animal origin, especially various beef products *(2, 16)*, as well as both raw *(7, 8)* and pasteurized milk *(17)*; however, they can also be isolated from yogurt *(10)* and unpasteurized apple cider *(1)* as well as drinking *(15)* and lake water *(3)*.

Low-temperature holding of foods (refrigeration at 5°C) was, until recently, the primary means utilized to restrict the growth of pathogens in foods. However, a group of pathogens capable of growth at 5°C, including *Listeria monocytogenes, Yersinia enterocolitica, Aeromonas hydrophila,* enterotoxigenic *E. coli, Clostridium botulinum* type E and nonproteolytic type B, is now recognized *(12)*. In addition, strains of *Bacillus (9)* and *Salmonella (4)* have been observed to grow at <10°C, temperatures which, though above the ideal holding conditions, are often encountered during the handling of many foods.

Recent work in this laboratory described the ability of certain strains of enterohemorrhagic *E. coli* to grow in broth culture at temperatures as low as 7.9°C and to produce verotoxin at these low temperatures *(13)*. The purpose of this study was to extend the earlier observations to foods by investigating the influence of temperature and background flora on the growth and verotoxin production of enterohemorrhagic *E. coli* in milk and ground beef.

MATERIALS AND METHODS

Cultures

The enterohemorrhagic *Escherichia coli* used in this study were O157:H7 (strains 90-1772 and 88-1558) and two nonmotile strains of O157 (H^-, designated NM1 and NM3) from the ERRC-MFS culture collection. The last two were chosen because of their ability to grow and form verotoxin at low temperatures *(13)*. Some experiments were performed using rifampin-resistant mutants of NM1 and NM3. These were isolated by streaking the cultures onto tryptic soy agar (TSA) (Difco Laboratories, Inc., Detroit, MI) containing 100 mg of rifampin (rifampicin, Sigma R-3501) (Sigma Chemical Co., St. Louis, MO) per liter. Preliminary experiments (data not shown) indicated that these mutants responded similarly to the parent strains. All cultures were maintained at 5°C in brain heart infusion broth (BHI) (Difco). Each culture was grown individually to stationary phase overnight in BHI broth at 37°C and diluted in 0.1% peptone (Difco) water for addition to the milk or ground beef.

Counting

The count of viable enterohemorrhagic *E. coli* was determined by surface plating (Spiral Plater Model D, Spiral Biotech, Bethesda, MD) onto selective media: sorbitol MacConkey agar (SMAC)

(Difco) or TSA containing 100 mg of rifampin per liter (TSAR). Serial dilutions were made as needed in 0.1% peptone water. In general, duplicate plates from each of the duplicate samples were analyzed. The background flora of the ground beef and milk was determined by surface plating onto TSA. Colonies were counted by a laser colony-counting system (Spiral Biotech) after incubation for 24 h at 37°C.

Foods

Milk. Milk, 2% fat, either pasteurized (local label) or ultrahigh temperature pasteurized (UHT-Long life, Parmalat brand, Moonachie, NJ), was purchased from a local supermarket. For the UHT pasteurized milk, viable cell counts on TSA (37°C for 24 h) at purchase and during storage at all temperatures always yielded ≤21 CFU/ml, the lower limit of detection. Raw whole milk was obtained from a local dairy farm from the holding tank immediately after milking, brought to the laboratory on ice and used within 24 h. The milks were inoculated with a dilution (made in 0.1% peptone water) of the individual *E. coli* strains to yield a starting count of ca. 10^3 CFU/ml. The inoculated milks were stored in sterile 50-ml polypropylene tubes (Falcon) and held at temperatures of 5, 8, 12, 15, and 37°C. At intervals appropriate to the holding temperature and the milk sample, 5-ml aliquots were removed and either plated directly or diluted as needed in 0.1% peptone water before plating.

Ground beef. Ground beef (80% lean) was obtained from a local supermarket. A portion was irradiated to an absorbed dose of 3 kGy at 5°C using a ^{137}cesium source to reduce the background microflora; this dose reduced the background flora from ca. 10^6 CFU/g to ca. 10^3 CFU/g, as determined by surface plating appropriate dilutions onto TSA.

The ground beef was inoculated with *E. coli* as follows: 125 g of ground beef was placed in a sterile plastic bag and inoculated with a dilution of an overnight culture (grown in BHI broth, stationary, 37°C) made in 0.1% peptone water to yield a starting count of ca. 10^3 CFU of *E. coli* per g. For controls, a similar amount of sterile 0.1% peptone water was added. Each ground beef sample was hand kneaded to evenly distribute the inoculum. Thirty-five grams of inoculated ground beef were then placed into individual sterile 50-ml polypropylene tubes (Falcon) and placed at 5, 8, 12, and 15°C.

Experimental design

Inoculated food was sampled at intervals appropriate to the specific temperature of storage and response anticipated. Duplicate portions of ground beef or milk were removed, added to 9 volumes (wt/wt) of sterile 0.1% peptone water, and mixed; appropriate dilutions were surface plated in duplicate with the Spiral Plater onto TSA or selective media. Colonies were counted after 24 h at 37°C. After the aliquots were removed for viable cell counts, the balance of the sample was processed for verotoxin determination as described below. All experiments were repeated at least twice; with similar responses observed. However, there were different sampling intervals in each experiment and no statistical comparisons were performed.

Verotoxin assay

Samples for verotoxin assays were taken from the 1/10 dilution used to determine viable cell count. Two ml of the slurry was centrifuged for 10 min at 5,000 rpm in an IEC centrifuge (International Equipment Co., Needham, Height, MA) at room temperature. The supernatant was then passed through an 0.2-μm-pore-size syringe filter (low protein binding) (Nalgene Co., Rochester, NY). Verotoxin assays were performed as described by Konowalchuk et al. *(6)* and Speirs et al. *(14)*. The concentration of suspended Vero cells was 10^5 to 10^6/ml; 100-μl aliquots of suspended Vero cells were placed into 96-well microtiter plates and incubated for 18 to 24 h at 37°C, 96% relative humidity, and 5% CO_2. Samples of the cell-free supernatant (100 μl) were placed in the first well of the plate and twofold dilutions were made across microtiter plates. After 72 h of incubation, the wells were examined for the presence of living and dead cells. The end point was the well (dilution) in which 50% of the Vero cells were dead and detached. The titer of verotoxin in a sample is expressed as the reciprocal of the end point dilution.

RESULTS AND DISCUSSION

This study extends our earlier work on the low-temperature growth of enterohemorrhagic *E. coli (13)* by investigating the influence of substrate and background flora on the response of four strains in foods. As suggested by Jay *(5)*, background flora was a major factor controlling the response of the strains, and the substrate (food matrix) appears also to be a factor.

The response of the four strains of *E. coli* in UHT-pasteurized milk at low temperatures is shown in Fig. 1. Strain 1772 grew well at 8°C and all four strains grew at 12°C, attaining maximum populations in 5 to 6 days, while at 15°C, maximum growth was observed by 3 days. These data indicate that care must be taken after opening containers of this type of product to keep it from being contaminated and to keep it cold. It should be noted that at 5°C the strains essentially maintained their numbers during the entire holding period and, at 8°C, the three strains which did not grow maintained their numbers during the holding period. These bacteria did remain viable in sterile (UHT-pasteurized) milk held at 5°C over the 12-day sampling period (data not shown). As expected, all four strains readily grew at 37°C, attaining a maximum population of 10^9 CFU/ml within 24 h (Fig. 1).

In pasteurized milk from the supermarket, the response of the strains was a function of the level of the background flora. When the background flora was at the same level as the *E. coli* (10^3 CFU/ml each), strain 1772 increased 3 log units

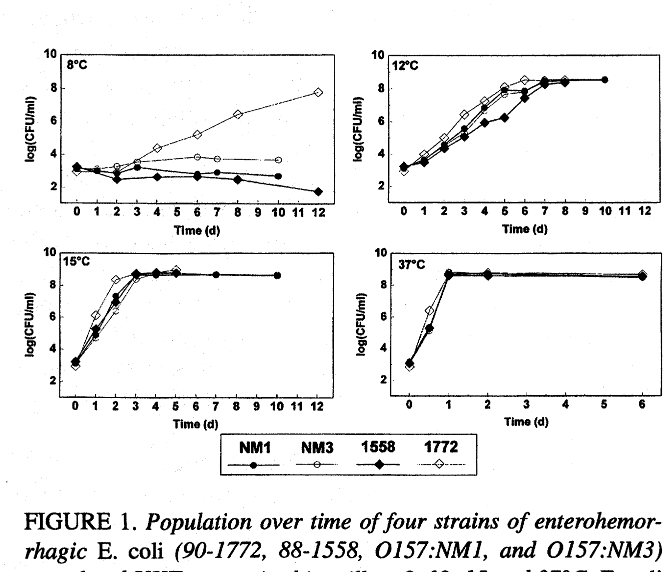

FIGURE 1. *Population over time of four strains of enterohemorrhagic* E. coli *(90-1772, 88-1558, O157:NM1, and O157:NM3) inoculated UHT-pasteurized in milk at 8, 12, 15 and 37°C:* E. coli *counts on sorbitol MacConkey agar.*

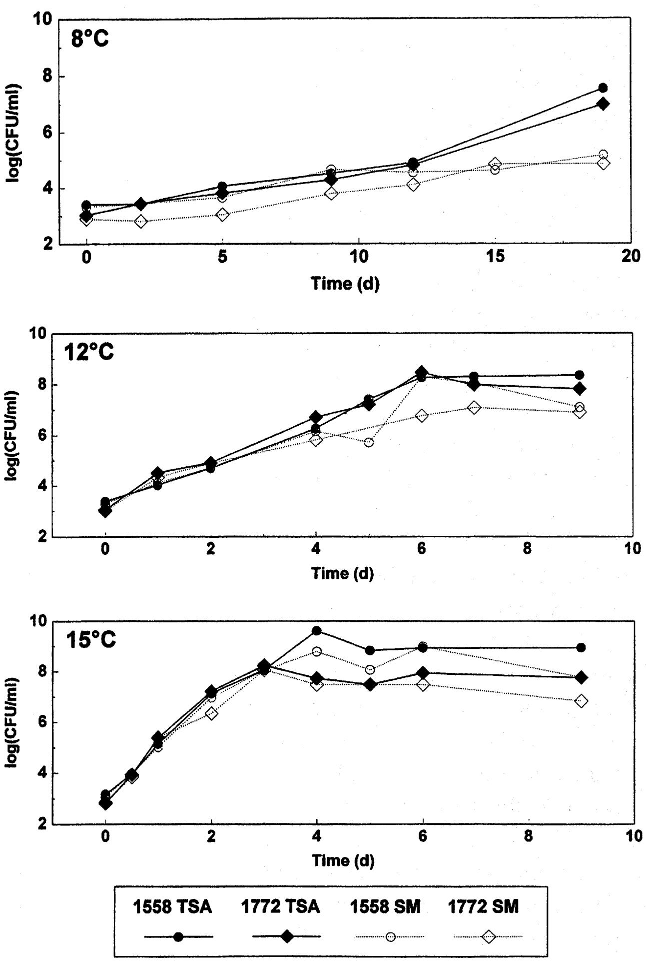

FIGURE 2. *Population over time of four strains of enterohemorrhagic* E. coli *(90-1772, 88-1558, O157:NM1, and O157:NM3) inoculated in low-background-flora pasteurized milk held at 8 and 12°C: total microbial count on TSA plates (closed symbols);* E. coli *counts on sorbitol MacConkey agar (SM, open symbols).*

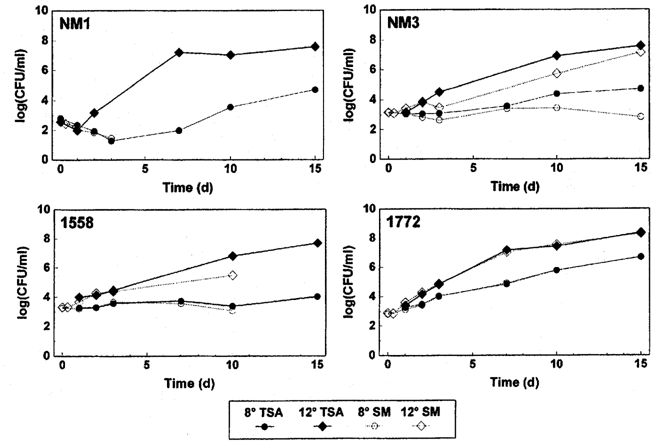

FIGURE 4. *Population over time of two strains of enterohemorrhagic* E. coli *(1558 and 1772) inoculated in irradiated ground beef (background flora reduced by a 3-kGy treatment at 5°C): total microbial count on TSA (closed symbols);* E. coli *count on sorbitol MacConkey agar (SM, open symbols).*

and strain NM1 about two logs (Fig. 2) at 8°C; at 12°C, all four strains grew. However, when the level of the background microflora was increased to 10^5 CFU/ml (milk was held for several days at 5°C before adding the *E. coli* strains), there was a smaller increase in cell numbers of strain 1772 at 8°C (Fig. 3). It should be noted that cell numbers of the other three strains remained constant when held at 8°C even in the presence of high numbers of the background flora.

When enterohemorrhagic *E. coli* was added to raw milk (starting count, 10^3 CFU/ml of both *E. coli* and background flora), the numbers of *E. coli* remained constant in milk held at 8 and 12°C (data not shown). The background flora rapidly increased in number, attaining 10^6 CFU/ml in 4 to 6 days.

In the first set of experiments, strains 1558 and 1772 were added to irradiated ground beef (starting background count of the background flora was the same as the starting

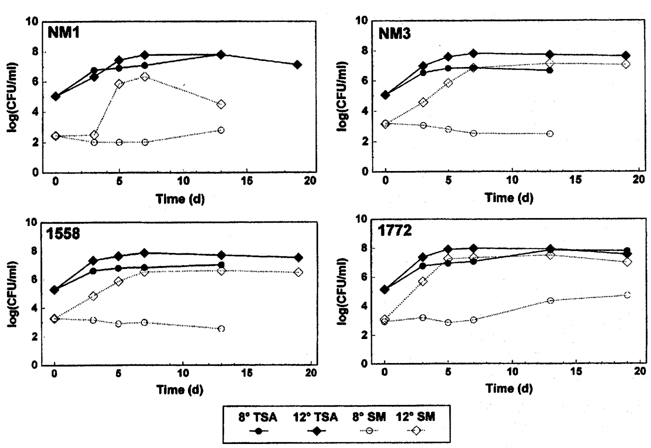

FIGURE 3. *Population over time of four strains of enterohemorrhagic* E. coli *(90-1772, 88-1558, O157:NM1, and O157:NM3) inoculated in high-background-flora pasteurized milk held at 8 and 12°C: total microbial count on TSA (closed symbols);* E. coli *counts on sorbitol MacConkey agar (SM, open symbols).*

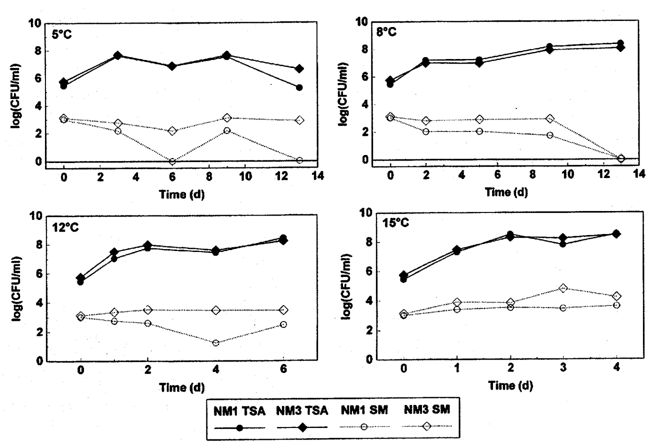

FIGURE 5. *Population over time of two rifampin-resistant strains of enterohemorrhagic* E. coli *(NM1 and NM3) inoculated in fresh ground beef held at 5, 8, 12, and 15°C: total count on TSA (closed symbols);* E. coli *count on selective medium (SM: TSA + rifampin, open symbols).*

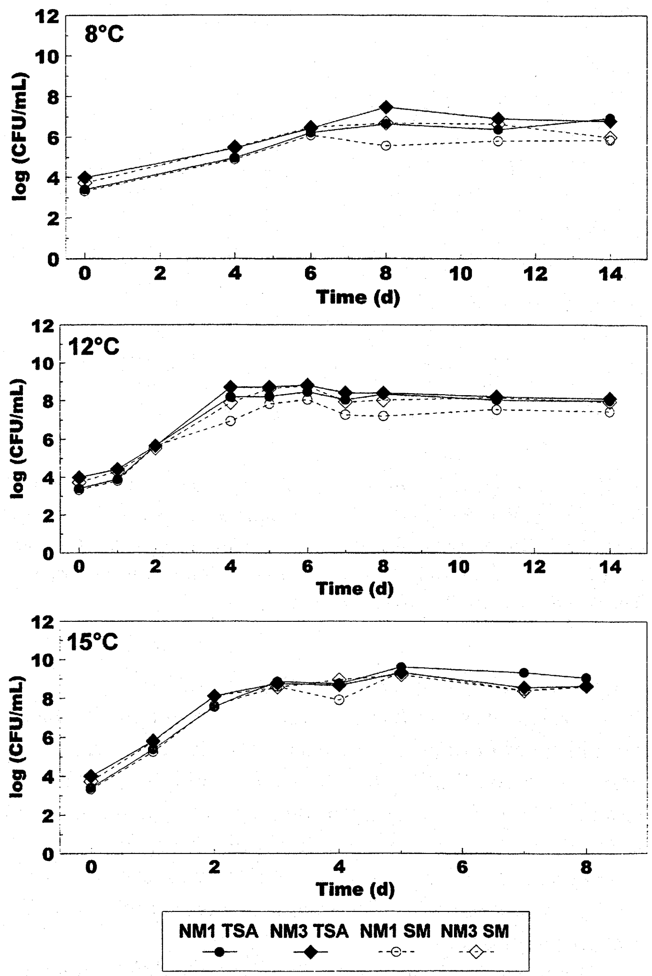

FIGURE 6. *Population over time of two rifampin-resistant strains of enterohemorrhagic* E. coli *(NM1 and NM3) inoculated in irradiated ground beef (background flora reduced by a 3-kGy treatment at 5°C) held at 8, 12, and 15°C: total count on TSA (closed symbols);* E. coli *count on selective medium (SM: TSA + rifampin, open symbols).*

count of *E. coli*), held at 8, 12, and 15°C, and counted at intervals. These data are presented in Fig. 4. As can be seen, counts of these two strains increased at all temperatures including 8°C, and in most instances, *E. coli* represented most of the flora in this meat (counts on TSA were similar to the *E. coli* counts).

Next, the response of the rifampin-resistant mutants of NM1 and NM3 was investigated in both fresh and irradiated ground beef. These strains were chosen because they were observed in a previous study to produce verotoxin in culture broth held at low temperatures *(13)*. The bacterial colonies were counted at intervals on TSAR. Their response to low temperature in fresh and irradiated ground beef is shown in Figures 5 and 6, respectively. While they did not grow in fresh ground beef held at 8°C, the viable count remained constant, i.e., they survived (Fig. 6). The strains behave similarly to 1558 and 1772 in the irradiated product (starting background count was the same as the starting *E. coli* count), i.e., they grew at 8°C (Fig. 6).

The formation of verotoxin in food has not been associated with foodborne illness; however, our earlier study *(13)* indicated that verotoxin was formed in culture broth at low temperatures. Thus, the potential exists for verotoxin production to occur in foods held at low temperatures. We investigated this possibility by testing milk and ground beef held at low temperatures. These results are shown in Table 1. The highest titer was 80 for strain NM1 in ground beef with a low population of background flora held at 15°C. Other strains in either milk or ground beef held at low temperatures produced lower verotoxin titers. Milk does not appear to be a very good substrate for verotoxin production, in that even at the optimum temperature of 37°C, strain NM3 yielded a titer of 320. This is in contrast to verotoxin production by the same strain in BHI broth which showed a very much higher titer at the same temperature (Table 1). The level of growth in the two substrates is comparable. Possible explanations are the presence of residual protease activity in the sterile milk and/or the presence of a compound(s) in sterile milk

TABLE 1. *Influence of temperature, strain, substrate, and background flora on verotoxin production by hemorrhagic* E. coli

Temperature (°C)	Substrate	Background flora	Strain	Verotoxin titer at holding time
37	UHT milk	none	NM3	320 at 24 h
15		none	NM3	40 at 4 days
15		none	NM1	20 at 4 days; 40 at 10 days
12		none	1558	20 at 4 days
12		none	NM1 & NM3	both were 20 at 7 days
8		none	all four strains	none detected at 12 days
12	Pasteurized milk	Start: *E. coli* 10^3 CFU/ml; background 10^5 CFU/ml	NM3	40 at 19 days
8–15	Ground beef	Start: *E. coli* 10^3 CFU/ml; background 10^6 CFU/ml	all four strains	none detected
15		Start: *E. coli* 10^3 CFU/ml; background 10^3 CFU/ml	NM1	80 at 8 days
12		Start: *E. coli* 10^3 CFU/ml; background 10^3 CFU/ml	NM1	20 at 14 days
8			NM1	20 at 14 days
15			NM3	20 at 6 days
12			NM3	20 at 8 days; 40 at 14 days
8			NM3	20 at 6 days
37	BHI broth	none	NM3	$>1.6 \times 10^{6}$ [a]

which inhibits verotoxin formation. Zucht et al. *(18)* reported the isolation and characterization of a 39-amino acid peptide with activity against *E. coli* and *Staphylococcus carnosus,* so there may be peptides present in milk which selectively inhibit verotoxin production.

In summary, the strains of enterohemorrhagic *E. coli* tested will grow at low temperatures in both milk and ground beef, as we found in our earlier observations of their responses in broth culture. Background flora of these foods, if present initially in numbers greater than the *E. coli,* will suppress this growth. However, the *E. coli* maintained itself when in nongrowth conditions. Some small amounts of verotoxin are formed in both milk and ground beef held at low temperatures, but even at the bacterium's optimum temperature (37°C), there was only a limited amount of verotoxin formed in milk. Our observations suggest that a food which becomes contaminated with enterohemorrhagic *E. coli* will remain a hazard even if the food is held at 5°C, i.e., under ideal refrigeration conditions: the cells, while not growing, remain viable.

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